RESEARCH ARTICLE

Novel antiosteoclastogenic activity of phloretin antagonizing RANKL-induced osteoclast differentiation of murine macrophages

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Scope: Bone-remodeling imbalance resulting in more bone resorption than bone formation is known to cause skeletal diseases such as osteoporosis. Phloretin, a natural dihydrochalcone compound largely present in apple peels, possesses antiphotoaging, and antiinflammatory activity.

Methods and results: Phloretin inhibited receptor activator of NF-κB ligand (RANKL)-induced formation of multinucleated osteoclasts and diminished bone resorption area produced during the osteoclast differentiation process. It was also found that ≥10 μM phloretin reduced RANKL-enhanced tartrate-resistance acid phosphatase activity and matrix metalloproteinase-9 secretion in a dose-dependent manner. The phloretin treatment retarded RANKL-induced expression of carbonic anhydrase II, vacuolar-type H^+-ATPase D2 and β3 integrin, all involved in the bone resorption. Furthermore, submicromolar phloretin diminished the expression and secretion of cathepsin K elevated by RANKL, being concurrent with inhibition of TRAF6 induction and NF-κB activation. RANKL-induced activation of nuclear factor of activated T cells c1 (NFATc1) and microphthalmia-associated transcription factor was also suppressed by phloretin.

Conclusion: These results demonstrate that the inhibition of osteoclast differentiation and bone resorption by phloretin entail a disturbance of TRAF6-NFATc1-NF-κB pathway triggered by RANKL. Therefore, phloretin may be a potential therapeutic agent targeting osteoclast differentiation and bone resorption in skeletal diseases such as osteoporosis.

Keywords:
Bone resorption / Nuclear factor κB / Osteoclastogenesis / Phloretin / Receptor activator of nuclear factor κB ligand

1 Introduction

Bone remodeling is a tightly regulated process to secure replacement of old bone with new bone through sequential osteoclastic resorption and osteoblastic bone formation [1]. In peripheral blood circulating precursor cells of osteoclasts and osteoblasts participating in the bone remodeling have been demonstrated. Preosteoblastic/stromal cells produce receptor activator of nuclear factor κB ligand (RANKL) and generate osteoprotegerin (OPG), a decoy receptor for RANKL [2–4]. OPG inhibits osteoclast differentiation and function by interrupting the interaction between RANKL and its receptor RANK [5]. The RANKL/OPG system is the dominant pathway regulating osteoclast recruitment and osteoclastogenesis in a stimulatory and inhibitory manner [6, 7]. Accordingly, the RANKL/OPG pathway has promise as a strategy for suppressing the excessive osteoclast formation characteristic of a variety of bone diseases.

Osteoclasts are multinucleated cells of macrophage origin that secrete lytic enzymes of acid hydrolases and proteases that degrade calcified bone matrix in a specialized extracellular compartment [8, 9]. The interaction of RANKL with RANK recruits adaptor molecules such as TNF receptor-associated factor 6 (TRAF6). The latter activates NF-κB, which is important for the initial induction of nuclear factor of activated T cells c1 (NFATc1) collaborating with other transcriptional

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Abbreviations: CA, carbonic anhydrase; **IκB**, inhibitory κB; **MITF**, microphthalmia-associated transcription factor; **MMP-9**, matrix metalloproteinase-9; **NFATc1**, nuclear factor of activated T cells c1; **RANKL**, receptor activator of nuclear factor-κB ligand; **TRAF**, tumor necrosis factor receptor-associated factor; **TRAP**, tartrate-resistant acid phosphatase; **V-ATPase**, vacuolar-type H^+-ATPase

partners to activate osteoclast-specific genes [4]. In addition, lipopolysaccharide and inflammatory cytokines such as TNF-α and IL-1 directly regulate osteoclast differentiation and function through a mechanism independent of the RANKL–RANK interaction. TGF-β and interferon-γ are important regulators in osteoclastogenesis [2]. The RANK signaling pathway of osteoclasts provides the molecular basis for developing therapeutics to treat osteoporosis and pathological diseases of bone loss [10].

Numerous studies have shown that natural polyphenols enhance osteoblastogenesis and prevent bone loss [11, 12]. Although green tea polyphenol (-)-epigallocatechin gallate (EGCG) inhibits osteoblast differentiation, this compound can reduce osteoclast formation and suppress bone resorption [13]. Phloretin is a natural dihydrochalcone compound rich in apple peels, possessing antitumor and antiphotoaging activities [14–16]. The apple polyphenol phloretin induces glutathione synthesis and heme oxygenase 1 and protects against oxidative stress through the extracellular signal-regulated kinase (ERK)/Nrf2 pathway [17]. In addition, phloretin present in apple juice extract reveals antiinflammatory activities by inhibiting proinflammatory gene expression [18]. However, there are no reports dealing with antiosteoporotic activity of phloretin. EGCG has an antiosteoclastogenic activity by attenuating RANKL induced the activation of JNK/c-Jun and NF-κB pathways [19]. Kaempferol antagonizes osteoclastogenic effects by disturbing osteoblast production and attenuating osteoclast precursor differentiation [20]. Dried plum polyphenols dampen osteoclastogenesis by reducing NFATc1 and inflammatory mediators [21]. Additionally, the inhibition of osteoclastogenesis can be accomplished by antiinflammatory and antioxidant agents [22]. Therefore, natural compounds and dietary components with antioxidant and antiinflammatory activity may block pathological bone loss and optimize bone health.

Based on literature regarding various physiological effects of natural compounds, this study investigated whether phloretin (Fig. 1A) exerted antiosteoclastogenic actions in murine RAW 264.7 macrophages cultured with RANKL. The activity of tartrate-resistant acid phosphatase (TRAP) and the induction of osteoclastogenic markers were examined in phloretin-treated differentiating RAW 264.7 macrophages. Additionally, the induction of bone-resorbing proteins and the bone resorption were determined. This study attempted to elucidate that phloretin manipulated the sequential molecular events induced by RANKL during osteoclast differentiation. The involvement of TRAF6-NF-κB signaling in the antiosteoclastogenesis of phloretin was examined.

2 Materials and methods

2.1 Materials

Fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA were purchased from Lonza (Walkersville, MD). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) was provided by DUCHEFA Biochemie (Haarlem, Netherlands). Minimum Essential Medium Alpha Medium (α-MEM), Dulbecco's modified eagle's media (DMEM), and phloretin were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Antibodies of mouse cathepsin K, mouse TRAF6, mouse phospho-inhibitory κB (phospho-IκB), and mouse vacuolar-type H^+-ATPase (V-ATPase) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse intergrin β3 was obtained from Cell Signaling Technology (Beverly, MA) and antimouse carbonic anhydrase (CA) II purchased from AbCam (Cambridge, UK). NF-κB inhibitor (SN50) was obtained from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase-conjugated goat antirabbit IgG was provided by Jackson ImmunoReserach Laboratories (West Grove, PA). RANKL was obtained from Peprotech (Rocky Hill, NJ). Phloretin was dissolved in dimethyl sulfoxide (DMSO) for live culture with cells; a final culture concentration of DMSO was <0.5%.

2.2 RAW 264.7 cell viability and osteoclast differentiation

Murine macrophage RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. For the osteoclast differentiation, cells were plated on 24-well plates at the density of 1×10^4 cells/well, and cultured for 5 d in α-MEM containing 10% FBS and 50 ng/mL RANKL in the absence and presence of 1–20 μM phloretin. Cell culture medium was changed every 2 d.

Cell proliferation and toxicity were determined by using a colorimetric assay based on the uptake of MTT by viable cells. After exposure to 1–20 μM phloretin for 2 d, cells were treated with 1 mg/mL MTT solution and incubated at 37°C for 3 h, resulting in the formation of blue formazan crystals. Absorbance of formazan was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm. Viability of RAW 264.7 cells was not influenced by treating 1–20 μM phloretin during 2-d differentiation (Fig. 1B), suggesting that ≤20 μM phloretin was not toxic to differentiating RAW 264.7 cells.

2.3 Measurement of TRAP activity

RAW 264.7 cells were fixed in 4% formalin for 10 min and stained with a commercial leukocyte acid phosphatase kit (Sigma-Aldrich Chemicals) according to the manufacturer's instructions. TRAP-positive multinucleated cells were visualized under light microscopy.

For measuring the TRAP activity, cells were fixed with 4% formalin for 10 min and 95% ethanol for 1 min. Subsequently, the dried cells were incubated in 10 mM citrate

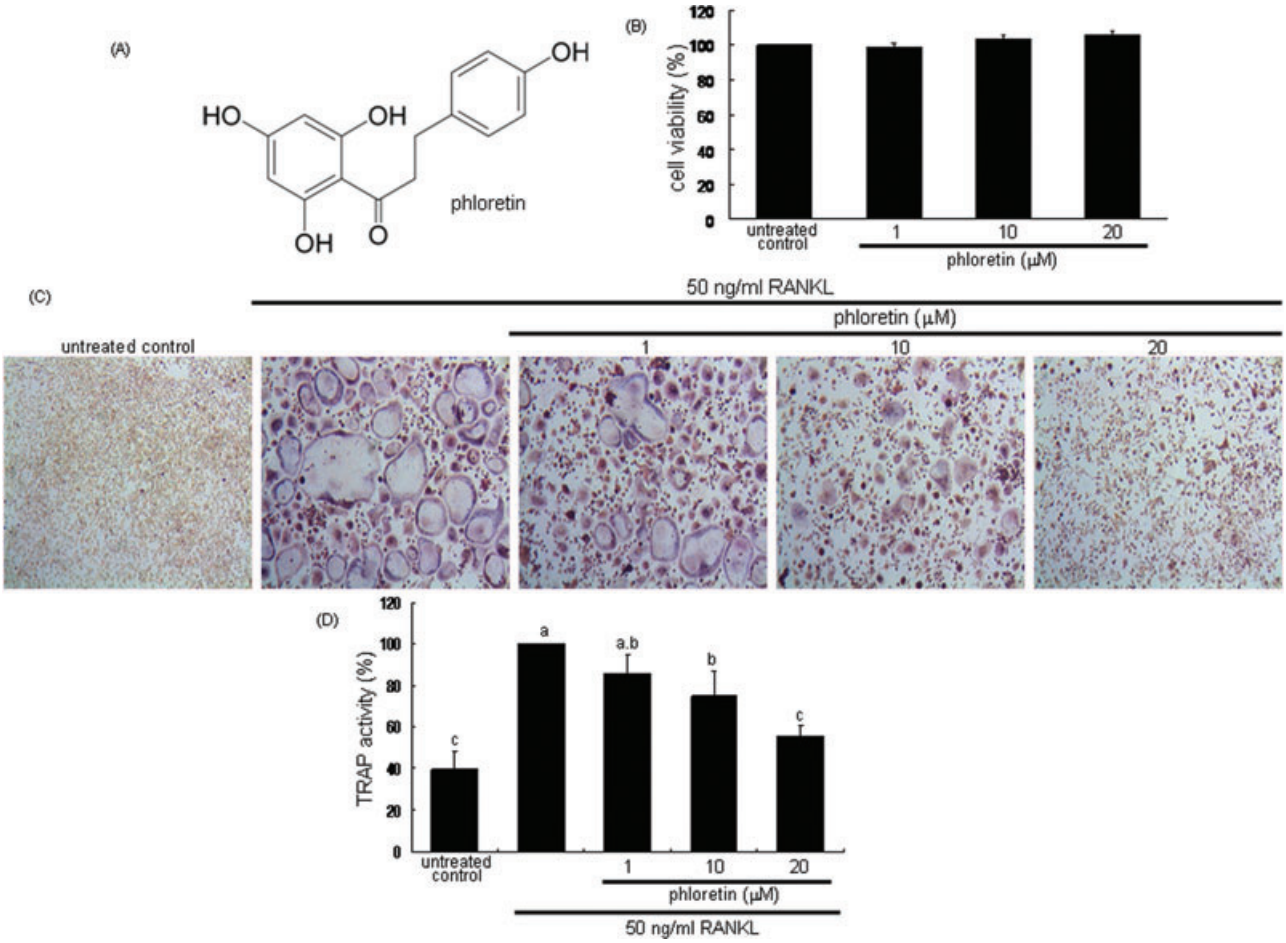

Figure 1. Chemical structure of phloretin (A), viability of RAW 264.7 cells (B), and suppression of osteoclast differentiation (C) and TRAP activity (D) by phloretin. RAW 264.7 cells were cultured for 2–5 d in the presence of 1–20 μM phloretin. After cells were cultured for 2 d with and without phloretin, cell viability was measured by MTT assay and values are means ± SEM ($n = 3$) and expressed as percent cell survival relative to phloretin-untreated cells (cell viability = 100%). After cells were cultured for 5 d, cells were fixed and stained using a leukocyte acid phosphatase kit, and TRAP-positive activity was determined. TRAP-positive multinucleated osteoclasts were visualized under light microscopy (three separate experiments). Magnification: 200-fold. TRAP activity was measured at $\lambda = 405$ nm. Values in bar graphs (means ± SEM, $n = 3$) not sharing a common letter indicate significant different at $p < 0.05$.

buffer (pH 4.6) containing 10 mM sodium tratrate and 5 mM *p*-nitrophenylphosphate. After incubation for 1 h, the reaction mixtures were transferred onto new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at $\lambda = 405$ nm by spectrophotometer, and the TRAP activity was expressed as percent of that of RANKL-untreated control.

2.4 Bone resorption assay

The osteoclast bone resorption assay was performed by using a commercially available bone resorption assay kit (CosMo Bio, Tokyo, Japan). RAW 264.7 cells were suspended in phenol red-free α-MEM containing 10% FBS, plated at a density of 1×10^4 cells/well and cultured for 5 d in the absence and presence of 50 ng/mL RANKL and 1–20 μM phloretin. To measure resorption pit areas, the cells were washed away in 6% NaOCl. The resorbed areas on the plate were visualized under light microscopy.

2.5 Gelatin zymography

RAW 264.7 cells plated at a density of 1×10^4 cells/well were incubated in phenol red-free α-MEM containing 10% FBS and 50 ng/mL RANKL in the absence and presence of 1–20 μM phloretin. After culture for 4 d, cells were starved for 24 h and culture media were collected. Gelatin zymography was performed to measure matrix metalloproteinase-9 (MMP-9) activity in culture media [23]. Culture media were subject to electrophoresis on 8% SDS-PAGE (300 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.03% bromophenol blue) co-polymerized with 0.1% gelatin. The gels were

incubated for 1 h in 2.5% Triton X-100 at 37°C, washed in 50 mM Tris-HCl buffer (pH 7.5) for 30 min, and incubated for 20 h in a buffer containing 200 mM NaCl, 10 mM $CaCl_2$, and 0.05% Brij-35. The gels were stained with 0.1% Coomassie Brilliant Blue G-250, 2% acetic acid and 45% methanol, and then destained in a solution with 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as unstained bands against the background of Coomassie blue-stained gelatin. The active form of MMP-9 was spotted as a band at 90 kDa in relation to the relative mobility of the marker proteins.

2.6 Western blot analysis

Western blot analysis was carried out using culture media and cell lysates prepared from cultured RAW 264.7 cells. Equal volumes of culture media and equal amounts of lysate proteins were electrophoresed on 6–12% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking membranes in a TBS-T buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl and 0.1% Tween 20) containing 3% bovine serum albumin or 5% nonfat milk for 3 h. The membranes were incubated with rabbit antimouse intergrin β3, rabbit antimouse cathepsin K, rabbit antimouse TRAF6, rabbit antimouse phospho-IκB, rabbit antimouse CA II, or rabbit antimouse V-ATPase as a primary antibody. The membranes were then incubated with goat antirabbit IgG conjugated to horseradish peroxidase as a secondary antibody. The protein levels on gels were determined by using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL), and Konica X-ray film (Konica, Tokyo, Japan). Incubation with monoclonal mouse β-actin antibody was conducted for comparative control.

2.7 Immunocytochemistry

After being stimulated with 50 ng/mL RANKL, RAW 264.7 cells grown on glass slides were thoroughly washed with PBS containing 0.2% Tween 20 (PBS-T), fixed with 4% ice-cold formaldehyde for 20 min, and made permeable with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. Cells were incubated for 1 h with 20% FBS to block any nonspecific binding. After washing with PBS-T, rabbit antimouse NF-κB p65 (Santa Cruz Biotechnology) was added to cells and incubated overnight at 4°C. Cells were washed with PBS-T and incubated with an FITC-conjugated goat antirabbit IgG as a secondary antibody. Fluorescent images were obtained by an Axiomager Optical fluorescent microscope (Zeiss, Germany).

2.8 Quantitative RT-PCR analysis

Total RNA was obtained from RAW 264.7 cells derived osteoclasts using a commercial Trizol reagent kit (Invitrogen, Carlsbad, CA). cDNA was synthesized using 5 μg of total RNA with 200 units of reverse transcriptase and 0.5 mg/mL oligo-$(dT)_{15}$ primer (Bioneer, Daejeon, Korea). The PCR (Bio-Rad Laboratories, Hercules, CA) was accomplished using mRNA transcripts of mouse NFATc1 (forward primer: 5′-CAACGCCCTGACCACCGATAG-3′, reverse primer: 5′-GGCTGCCTTCCGTCTCATAGT-3′, 392 bp), mouse microphthalmia transcription factor (MITF) (forward primer: 5′-CTGATCTGGTGAATCGGATC-3′, reverse primer: 5′-TCCTGAAGAAGAGAGGGAGC-3′, 390 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward primer: 5′-AACTTTGGCATTGTGGAAGGG-3′, reverse primer: 5′-GACACATTG GGGGTAGGAACAC-3′, 224 bp) with an addition of 25 μL of 10 mM Tris-HCl (pH 9.0) containing 25 mM $MgCl_2$, 10 mM dNTP, and 5 units of Taq DNA polymerase. Each cycle consisted of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, and the final extension was for 10 min at 72°C. After thermocycling and electrophoresis of the PCR products (10 μL) on 1% agarose gel containing 0.5 mg/mL ethidium bromide, the bands were visualized using a TFX-20 M model-UV transilluminator (Vilber-Lourmat, Marne-la-Vallée, France) and gel photographs were obtained. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

2.9 Statistical analyses

The results were expressed as means ± SEM for each treatment group in each experiment. Statistical analyses were performed using Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC). Significance was determined by one-way analysis of variance, followed by Duncan range test for multiple comparisons. Differences were considered significant at $p < 0.05$.

3 Results

3.1 Inhibitory effects of phloretin on osteoclast differentiation

Phloretin was not toxic to RAW 264.7 macrophages during 2-day differentiation (Fig. 1B). Signaling by RANKL is crucial for terminal differentiation of macrophages into osteoclasts [5, 6, 8]. This study attempted to examine that RANKL differentiated RAW 264.7 macrophages to TRAP-positive multinucleated cells, which was disturbed by phloretin. There was no detectable TRAP staining observed in undifferentiated cells (Fig. 1C). However, 50 ng/mL RANKL produced numerous TRAP-positive multinucleated cells. When 1–20 μM phloretin was treated for 5 d, TRAP-positive multinucleated cells disappeared. We also examined the inhibitory effect of nontoxic phloretin on TRAP activity of RAW 264.7 cells stimulated with 50 ng/mL RANKL (Fig. 1D). RANKL fostered TRAP

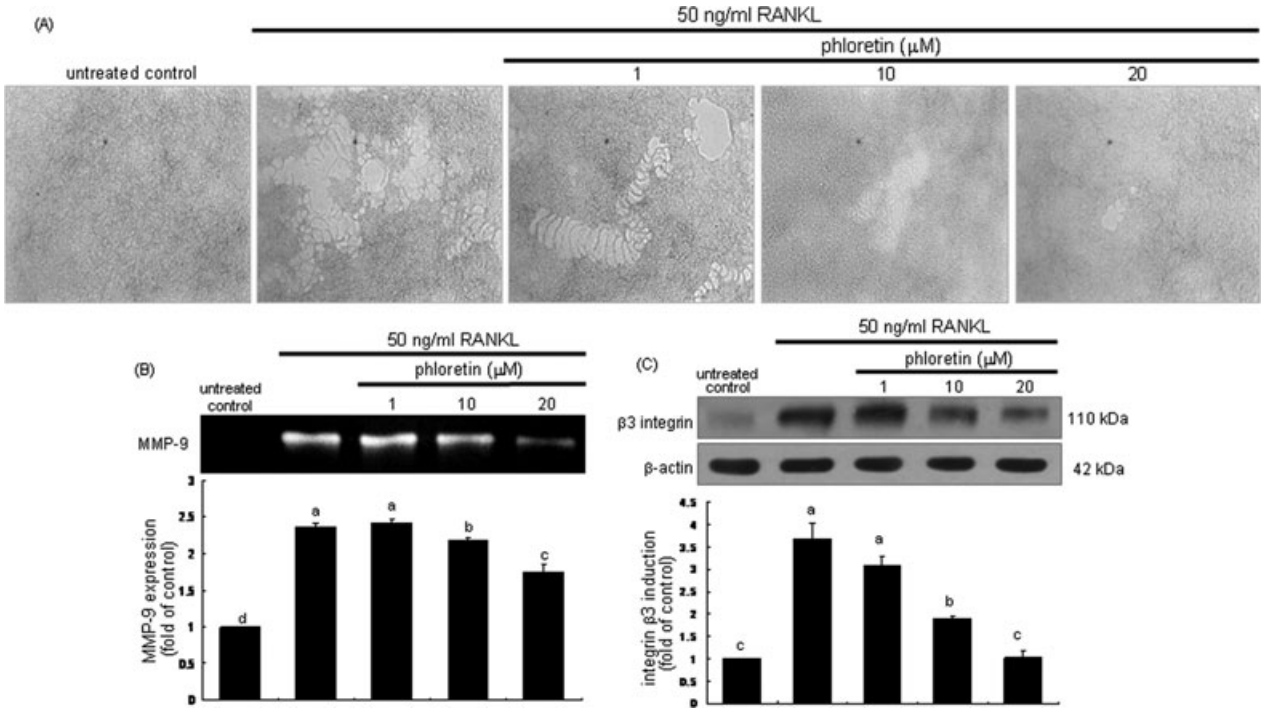

Figure 2. Inhibition of bone resorption (A), MMP-9 secretion (B), and β3 integrin induction (C) by phloretin. Osteoclast bone resorption assay was performed by using a commercial kit. RAW 264.7 cells were cultured in serum free α-MEM with 50 ng/mL RANKL for 5 d. Resorbed areas on the plate were visualized under light microscopy (A, three separate experiments). Magnification: 200-fold. To measure RANKL-induced MMP-9 secretion for 24 h, gelatin zymograph was performed (B). Gelatinolytic activity was detected as unstained against the background of Coomassie blue-stained gelatin. Cell lysates were subject to SDS-PAGE and Western blot analysis with a primary antibody against β3 integrin (C). Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) represent quantitative results of the upper bands obtained from a densitometer. Values not sharing a common letter are significantly different at $p < 0.05$.

activity in RAW 264.7 macrophages. In contrast, this activity dose-dependently declined in phloretin-treated macrophages exposed to RANKL.

3.2 Inhibition of bone resorption by phloretin

The inhibitory effect of phloretin on bone resorption was tested in RANKL-differentiated cells cultured on well plates coated with calcium phosphate for 5 d. RANKL treatment augmented the number and size of bone resorption areas in osteoclasts transformed from RAW 264.7 cells (Fig. 2A). However, phloretin mitigated bone resorption activity in a dose-dependent manner.

MMP-9 is responsible for the bone resorption mediated by mature osteoclasts [24]. To test with gelatin zymography whether phloretin encumbered RANKL-induced MMP-9 secretion, RAW 264.7 cells were exposed to 50 ng/mL RANKL for 2 d under serum-free conditions. RANKL considerably elevated cellular secretion of MMP-9, which was reversed by phloretin (Fig. 2B).

It is known that αvβ3 integrin plays a role in the regulation of cell migration and maintenance of the sealing zone required for the effective osteoclastic bone resorption [25]. Cellular β3 integrin was elevated by 50 ng/mL RANKL treatment for 5 d. In contrast, phloretin alleviated its induction in a dose-dependent manner (Fig. 2C)

3.3 Suppression of cathepsin K production of osteoclasts by silibinin

Osteoclast-specific cathepsin K is elevated during maturation. Cathepsin K is secreted in sealed zone beneath the ruffled border of the osteoclast and plays a pivotal role in bone resorption [26]. RANKL enhanced secretion of cathepsin K from RAW 264.7 macrophages and such secretion was significantly attenuated by treating ≥10 μM phloretin (Fig. 3A). In addition, the RANKL-induced cellular expression of cathepsin K was down-regulated by presence of phloretin (Fig. 3B). Accordingly, phloretin was effective in retarding bone resorption and osteoclasts maturation.

3.4 Down-regulation of TRAF6 and NF-kB transactivation by phloretin

RANKL induces activation of NF-κB signaling pathway during osteoclast differentiation [4]. When RAW 264.7 cells

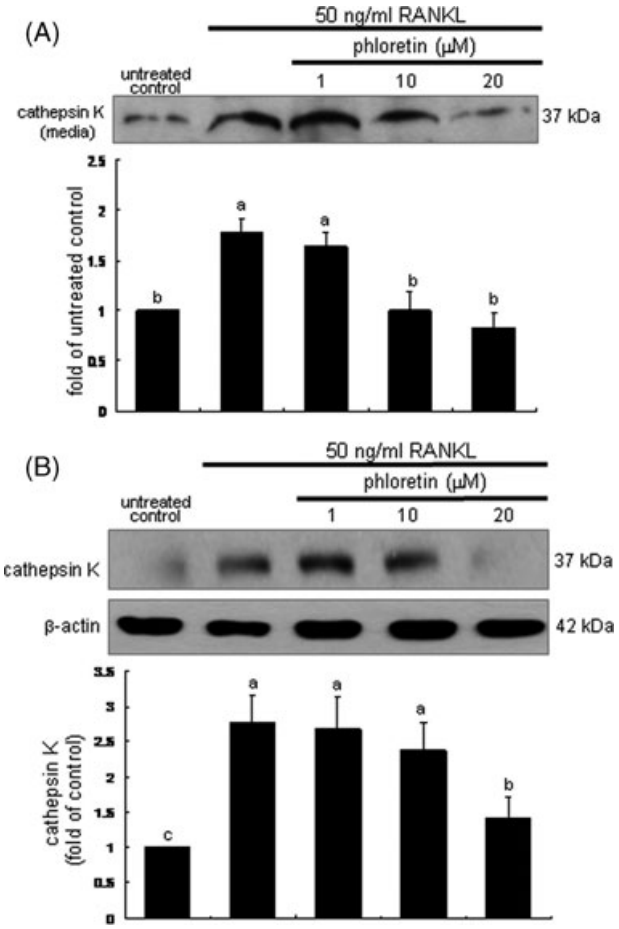

Figure 3. Western blot data showing phloretin inhibition of RANKL-induced cathepsin K secretion (A) and cellular expression (B). RAW 264.7 cells were cultured in α-MEM and exposed to 50 ng/mL RANKL for 5 d in the absence and presence of 1–20 μM phloretin. Culture media and cell lysates were subject to SDS-PAGE and Western blot analysis with a primary antibody against cathepsin K. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the bottom panels represent quantitative results of blots obtained from a densitometer. Values not sharing a common letter are significantly different at $p < 0.05$.

were treated with 50 ng/mL RANKL, the TRAF6 expression attained to the peak on 30 min (data not shown). When cells were incubated with 1–20 μM phloretin, the TRAF6 induction was notably diminished (Fig. 4A).

RANKL enhanced IκB phosphorylation leading to nuclear translocation of NF-κB (Fig. 4B), which was diminished in cells supplemented with ≥10 μM phloretin. As expected, cytoplasmic immunofluorescence staining was observed in untreated controls. RANKL increased nuclear staining with specific anti-NF-κB p65 at single cell level, indicative of nuclear localization of activated NF-κB (Fig. 4C). However, phloretin-treated cells diminished staining level of nuclear NF-κB p65 and cytoplasmic immunofluorescence staining was observed.

As shown in Fig. 5A, as with 20 μM phloretin, the NF-κB inhibitor SN50 inhibited the formation of TRAP-positive multinucleated cells by RANKL. In addition, the RANKL-induced cathepsin K production was suppressed by 10 μM SN50 (Fig. 5B). Accordingly, phloretin may limit cellular secretion of TRAP and cathepsin K via disturbing an NF-κB-responsive mechanism

3.5 Suppression of CA II and V-ATPase D2 induction of osteoclasts by phloretin

CA II and V-ATPase subunits are expressed in high amounts in resorbing osteoclasts and osteoclast precursors [26]. V-ATPase located in the osteoclast ruffled border membrane, maintains a low pH in the extracellular lacuna [27]. Protons for this V-ATPase are generated by a cytoplasmic CA II. RANKL-induced expression of CA II and V-ATPase D2 from RAW 264.7 macrophages treated with 50 ng/mL RANKL (Fig. 6). Such induction was significantly attenuated by treating ≥10 μM phloretin. Accordingly, phloretin was effective in retarding active bone resorption in a sealed compartment between the ruffled border and the bone surface.

3.6 Blockade of RANKL-induced transcription of NFATc1 and MITF by phloretin

NFATc1 has been characterized as a master regulator of NF-κB ligand-induced osteoclast differentiation [28]. MITF is a master regulator of osteoclast bone resorption and a key regulator of osteoclast function by activating genes such as TRAP and cathepsin K [29]. When RAW 264.7 cells were exposed to 50 ng/mL RANKL for 2 d, the transcription of NFATc1 and MITF was induced (Fig. 7). In contrast, ≥10 μM phloretin attenuated the up-regulated mRNA levels of NFATc1 and MITF. Thus, phloretin may antagonize osteoclast function promoting osteoclastogenic genes of TRAP and cathepsin K.

4 Discussion

Seven major findings were extracted from this study. (i) Dietary compound phloretin, a natural dihydrochalcone present in apple peels, inhibited the RANKL-induced differentiation of RAW 264.7 macrophages into multinucleated osteoclasts. (ii) The apple polyphenol phloretin diminished the bone resorption promoted by RANKL during RAW 264.7 macrophage differentiation. (iii) Phloretin nontoxic at 1–20 μM dose-dependently reduced the osteoclast differentiation-enhanced TRAP activity, MMP-9 secretion, and β3 integrin expression. (iv) Submicromolar phloretin mitigated the production of cathepsin K promoted by RANKL. (v) The phloretin treatment attenuated RANKL induction of CA II and V-ATPase D2, all responsible for bone resorption. (vi) The inhibition of TRAF6 induction and NF-κB activation

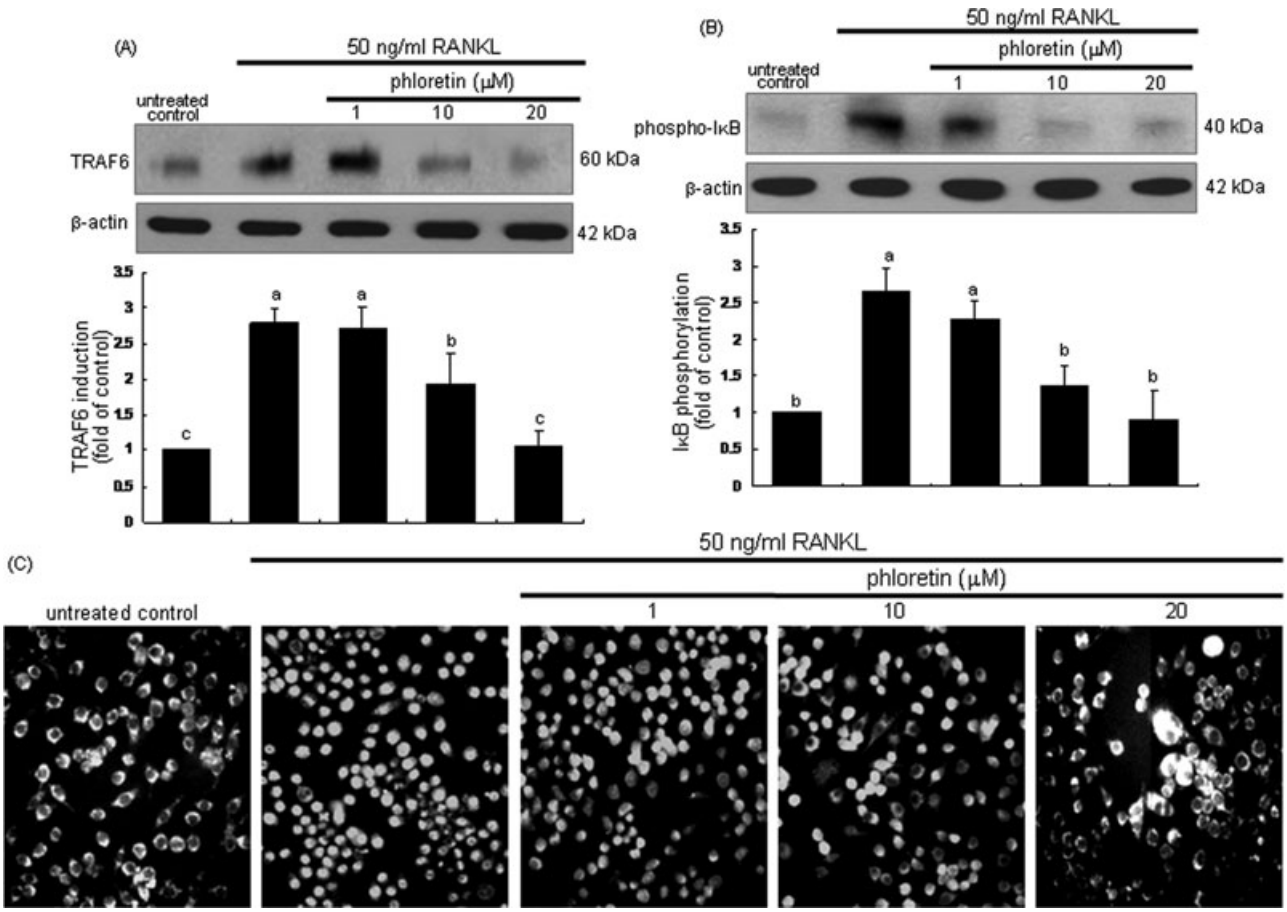

Figure 4. Phloretin inhibition of RANKL-induced TRAF6 expression (A), IκB phosphorylation (B), and NF-κB nuclear translocation (C). RAW 264.7 cells were cultured for 30 min in the absence and presence of 1–20 μM phloretin. After cells extracts were subject to SDS-PAGE and Western blot analysis with a primary antibody against TRAF6 and phospho-IκB (A and B). Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the bottom panels represent quantitative results obtained from a densitometer. Values in bar graphs not sharing a letter indicate significant different at $p < 0.05$. Immunocytochemical analysis (C) showing inhibition of RANKL-induced NF-κB translocation by phloretin. NF-κB localization was visualized with an FITC-conjugated secondary antibody. Original magnification of microscopic images ($n = 3$), 200×.

was observed in phloretin-treated osteoclasts. (vii) RANKL-induced transcription of NFATc1 and MITF was demoted by phloretin. These observations demonstrate that nontoxic phloretin exerted osteoprotective effects by blunting osteoclast differentiation and osteoclastic bone resorption through disturbing TRAF6-NF-κB-NFATc1/MITF signaling pathway. Accordingly, phloretin was a potential therapeutic agent encumbering osteoclastogenesis.

Bone remodeling is a tightly regulated process to secure normal repair of skeletal damage and replacement of old bone with new one. The elimination of old bone by osteoclasts is sequentially followed by the formation of mineralized bone matrix by osteoblasts [1, 30]. The imbalance of bone remodeling takes place due to bone resorption excessive for bone formation and leads to causing skeletal diseases such as osteoporosis [30]. Inhibiting RANKL production of osteoblasts or antagonizing RANKL actions on osteoclasts might be a protective mechanism against excessive osteoclast differentiation. Encumbering RANK signaling pathway might be therapeutics to treat pathological bone loss diseases [10]. This study elucidated whether the natural compound phloretin antagonized RANKL actions responsible for osteoclastogenesis. Phloretin blunted osteoclast differentiation of RAW 264.7 macrophages by attenuating TRAP activity.

Natural polyphenols have potentials to antagonize osteoclastogenic effects and bone resorption by suppressing osteoblast differentiation and production of osteoclastogenic cytokines [13, 20]. Several reports have demonstrated antioxidant and antiinflammatory properties of phloretin [17, 18]. However, there are no reports regarding antiosteoporotic or antiosteoclastogenic activity of phloretin. Accordingly, the osteoprotective effects of phloretin on bone loss remain to be elucidated. The present study revealed that phloretin inhibited RANKL-induced osteoclastogenesis by dampening the secretion of the proteolytic enzymes of MMP-9 and cathepsin K. Consequently, phloretin inhibited the degradation

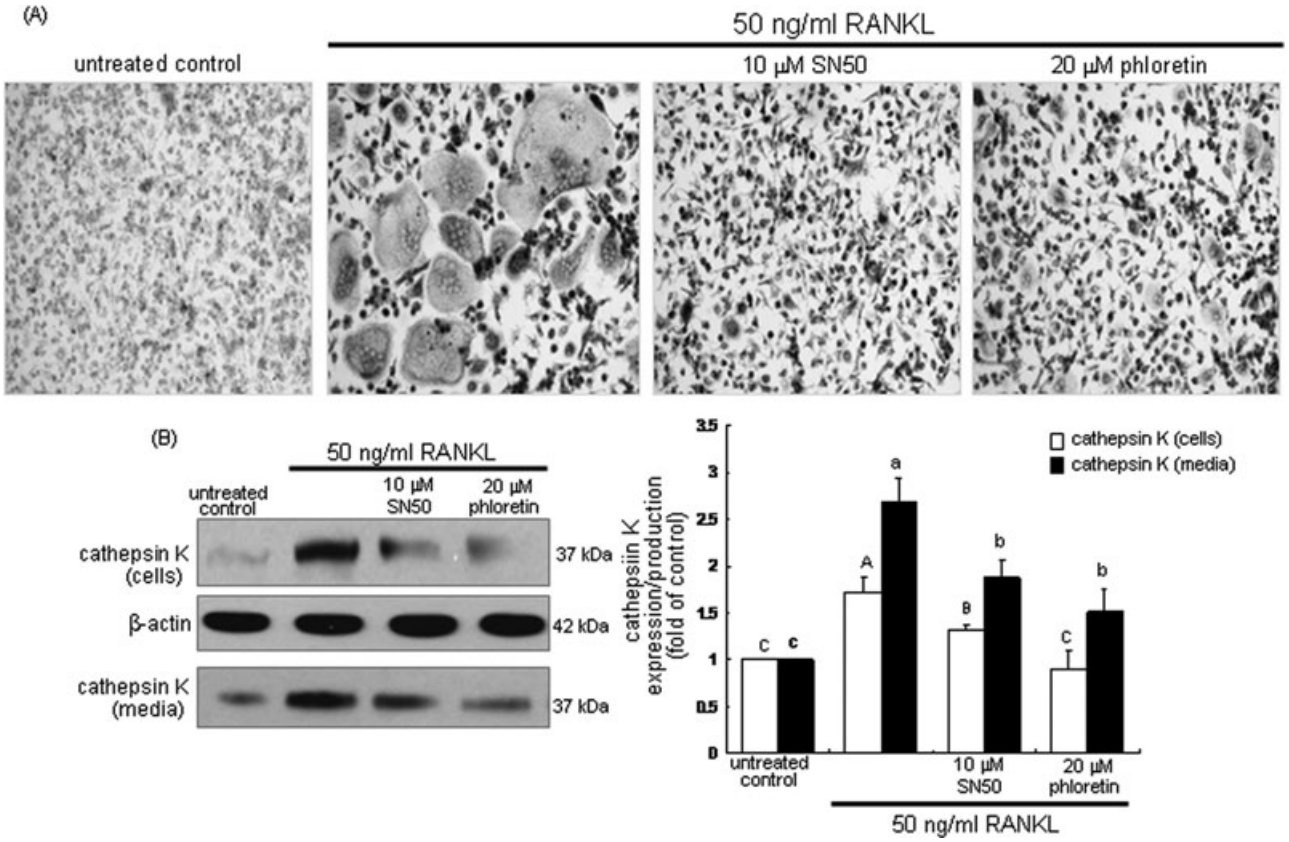

Figure 5. Inhibitory effects of NF-κB inhibitor SN50 on RANKL-induced osteoclast differentiation (A) and cathepsin K production (B). RAW 264.7 cells (1×10^4 cells/well) were cultured in α-MEM and exposed to 50 ng/mL RANKL for 5 d in the absence and presence of 10 μM SN50 or 20 μM phloretin. Cells were fixed and stained using a leukocyte acid phosphatase kit (A). TRAP-positive multinucleated osteoclasts were visualized under light microscopy (three separate experiments). Magnification: 200-fold. Culture media and cell extracts were subject to SDS-PAGE and Western blot analysis with a primary antibody against cathepsin K. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the right panels represent quantitative results obtained from a densitometer. Respective means without a common letter differ, $p < 0.05$.

of organic matrix occurring in sealed lacunae beneath the ruffled border of osteoclasts. However, the in vivo role as an osteoprotective agent is not yet defined. Daily intake of the apple polyphenol phloridzin prevented ovariectomy- and inflammation-induced bone loss in rats by improving inflammatory markers and bone resorption [31].

Active bone resorption involves acidification, coincident with production of protons in the cytoplasm by CA II and their subsequent transport through the ruffled border into the resorption lacuna by an osteoclast-specific V-ATPase. In this study, phloretin suppressed the induction of CA II and V-ATPase D2 by RANKL, thereby abrogating acidification and demineralization of the bone matrix of sealed lacunae. In addition, phloretin diminished the β3 integrin induction in RANKL-matured osteoclasts. The vitronectin receptor of αvβ3 integrin plays a role in the regulation of cell migration and maintenance of the sealing zone required for the effective osteoclastic bone resorption [32]. Phloretin disabled mature osteoclasts from migrating onto bone surface destined for resorption and preserving the sealing zone. Thus, it can be speculated that dietary compound phloretin is a novel agent treating bone disorders involving excessive resorption of mature osteoclasts. There are few investigations with natural compounds on the inhibition of osteoclastic acidification. A natural compound diphyllin potently inhibits V-ATPase and lysosomal acidification in osteoclasts leading to bone resorption [33].

Dried plum polyphenols dampen osteoclastogenesis by down-regulating NFATc1 and inflammatory mediators such as nitric oxide and TNF-α [21]. The current study attempted to explore the precise inhibitory mechanism(s) by which apple polyphenol phloretin abrogated osteoclastogenesis and bone resorption. It is deemed that the inhibition of osteoclastogenesis by phloretin may be partially attributed to its antiinflammatory and antioxidant properties. Based on the antiinflammatory activity of phloretin [17], the effects of phloretin on NF-κB signaling were elucidated. This study found that phloretin inhibited TRAF6 expression and NF-κB transactivation in RANKL-treated RAW 264.7 macrophages. Finally, phloretin encumbered TRAF6-NF-κB-dependent

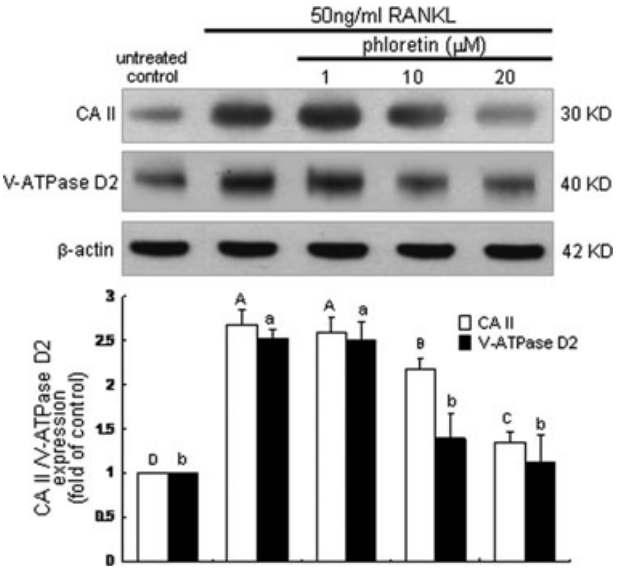

Figure 6. Suppressive effects of phloretin on RANKL-induced cellular expression of CA II and V-ATPase D2. RAW 264.7 cells were cultured in α-MEM and exposed to 50 ng/mL RANKL for 5 d in the absence and presence of 1–20 μM phloretin. Cell extracts were subject to SDS-PAGE and Western blot analysis with a primary antibody against CA II and V-ATPase D2. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the bottom panels represent quantitative results obtained from a densitometer. Respective means without a common letter differ, $p < 0.05$.

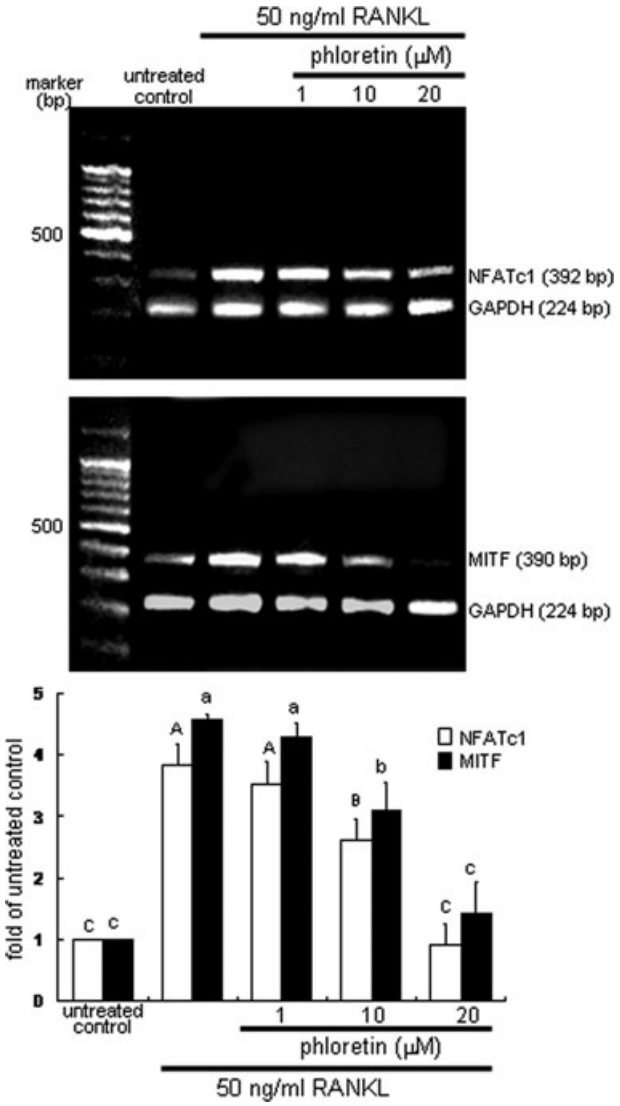

Figure 7. Attenuation of NFATc1 and MITF transcription in phloretin-treated osteoclasts. NFATc1 and MITF mRNA levels of osteoclasts were determined by using RT-PCR analysis. RAW 264.7 cells were cultured in α-MEM and exposed to 50 ng/mL RANKL for 2 d in the absence and presence of phloretin. GAPDH gene was used as an internal control for the co-amplification with NFATc1 and MITF (three separate experiments). The bar graphs (means ± SEM, $n = 3$) represent quantitative results obtained from a luminometer. Respective means without a common letter differ, $p < 0.05$.

transcriptional signaling in response to RANKL stimulation. The disruption of TRAF6 by phloretin impaired osteoclast bone resorbing activity. Naringin perturbs osteoclast formation and bone resorption by suppressing gene expression of key osteoclast marker genes through the inhibition of RANK-mediated NF-κB and ERK signaling [34]. It was found in the experiments with SN50 that phloretin mitigated the secretion of TRAP and cathepsin K by deterring NF-κB transactivation.

The initial induction of the transcription factor NFATc1 activated by calcium signaling entails NF-κB signaling [4]. NFATc1 collaborates with other transcriptional partners such as activator protein-1/c-Fos complex to activate osteoclast-specific genes. Resveratrol inhibits RANKL-induced osteoclast differentiation, cathepsin K gene induction, and bone resorption, which is associated with decreased NFATc1 stimulation and NF-κB nuclear translocation [35]. Similarly, phloretin attenuated NFATc1 transcription followed by NF-κB transactivation. This finding suggested that phloretin blunted RANKL-induced osteoclastogenesis by inhibiting the secretion of TRAP and cathepsin K responsive to the TRAF6-NF-κB-NFATc1 signaling. In addition, MITF-specific genes during osteoclast activation may be considered therapeutic targets for the treatment of bone resorption disorders. Phloretin suppressed the MITF transcription, whose phenomenon appeared to play an important role during osteoclastogenesis. MITF functions cooperatively with NFATc1 to transactivate the V-ATPase D2 promoter during RANKL-induced osteoclastogenesis [36]. Accordingly, this study speculated that phloretin antagonized cathepsin K proteolytic activation and V-ATPase D2-associated acidification leading to demineralization of bone matrix by inhibiting NFATc1 and MITF transcription.

In summary, the current report demonstrated that phloretin abrogated RANKL-induced osteoclastogenesis by retarding osteoclast differentiation and bone resorption. Phloretin diminished MMP-9 activity and inhibited cellular induction of β3 integrin and cathepsin K. Furthermore, phloretin mitigated the TRAF6 expression and inhibited the NF-κB transactivation concurrently with demoting the transcription of NFATc1 and MITF. Accordingly, the inhibition of osteoclast differentiation and bone resorption by phloretin was achieved by disturbing TRAF6-NF-κB-NFATc1 signaling pathway. Although phloretin may serve as a modulator against osteoporosis and pathological bone loss under in vitro conditions, the role as an osteoprotective agent remains indistinct in clinical point of view.

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